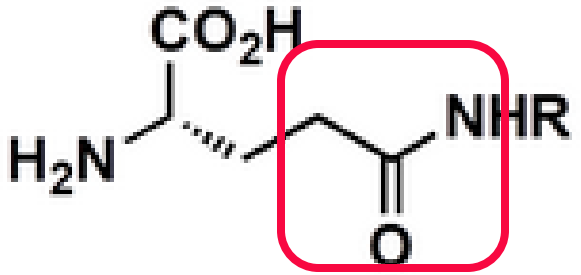
Sustainable production of L-theanine, a nutraceutical, using microbial gamma-glutamyl transpeptidase

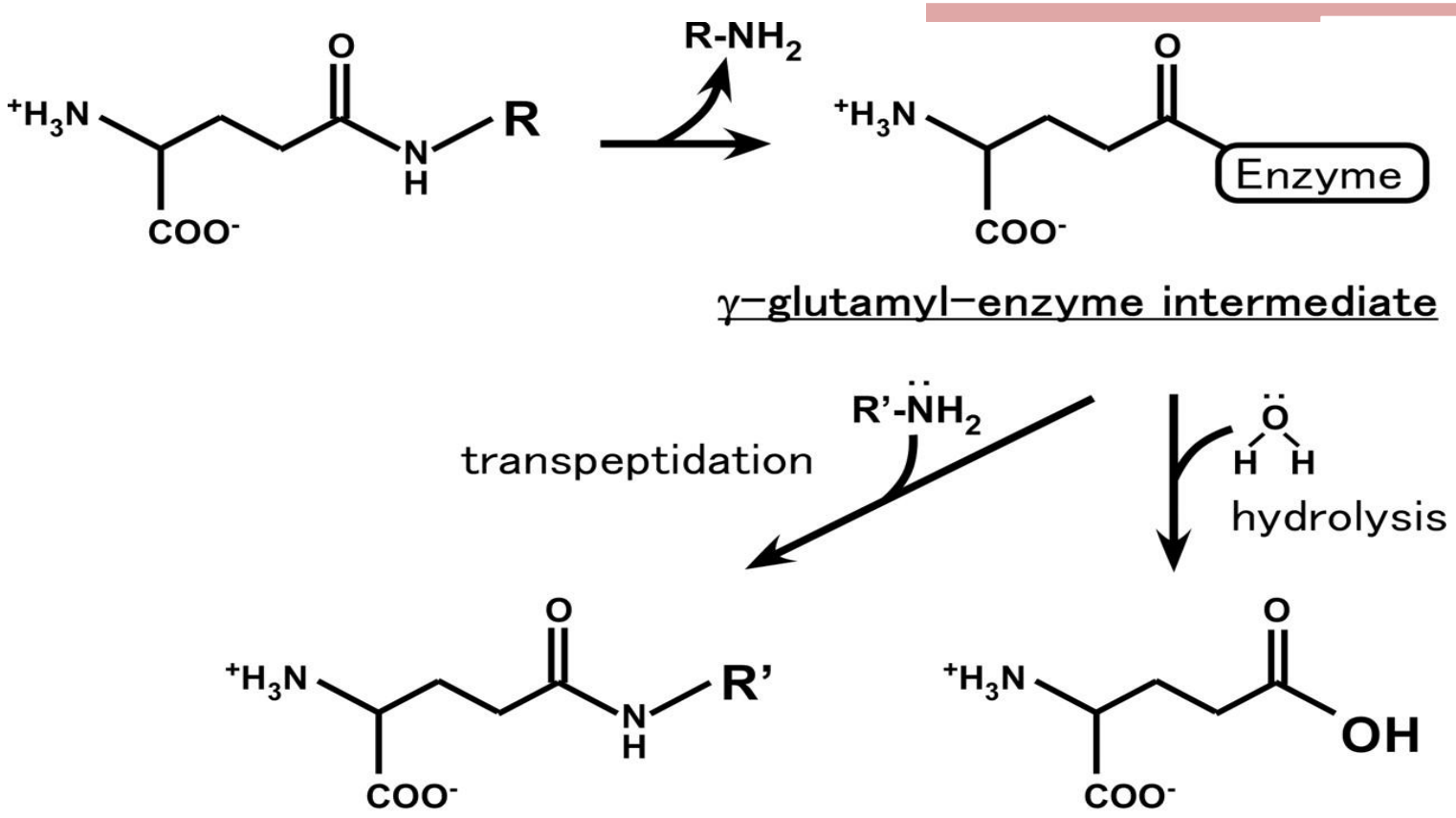
Shruti B. Rajput

Department of Microbiology
University of Delhi
South Campus

Γ-glutamyl transpeptidase

- Γ-glutamyl transpeptidase (GGT; E.C.2.2.3.2) is ubiquitously distributed in bacteria, yeast, plants and in animals from nematodes to humans (Rawlings *et al.*, 2006).
- Belongs to N-terminal nucleophile (Ntn) hydrolases super-family.
- Unique transpeptidase which cleaves **γ-bond** as well as transfers **γ-glutamyl moeity to an acceptor.**



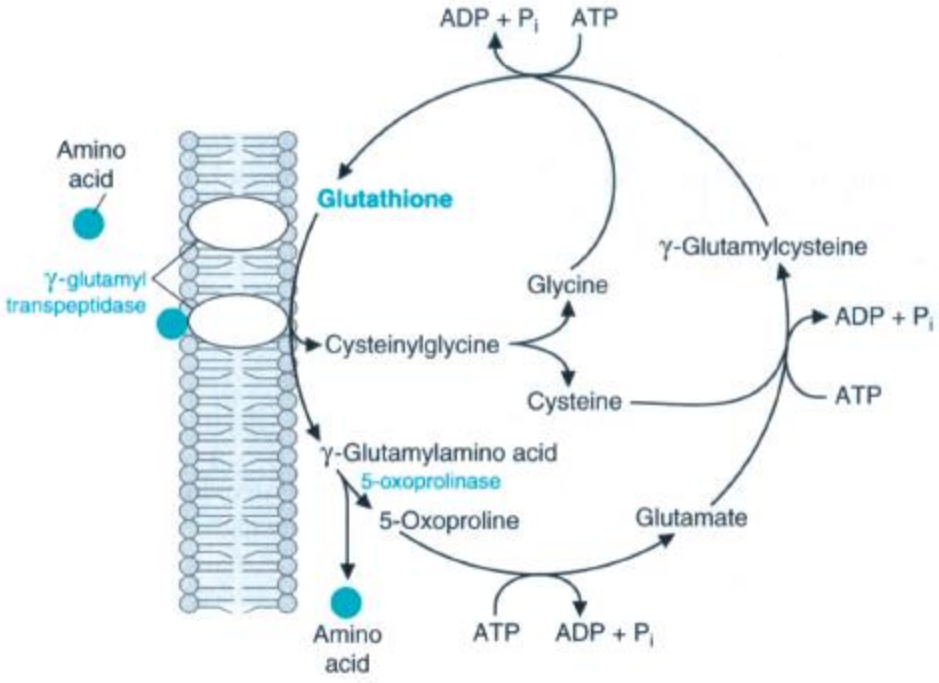


- It is a two substrate enzyme that removes the terminal γ-glutamyl residue from a molecule of the general form Glu-γCO-NH-R by breaking the amide bond and transfers it to water (hydrolysis), amino acids, or peptides (transpeptidation).

- It follows a ping pong mechanism for catalyzing the above reaction.

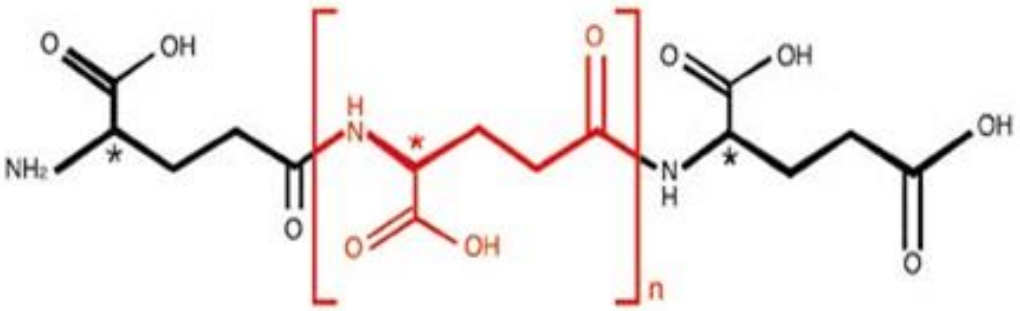
Role in Cell physiology

- In mammals, GGT is a transmembrane enzyme and catalyses the first reaction of γ-glutamyl cycle.

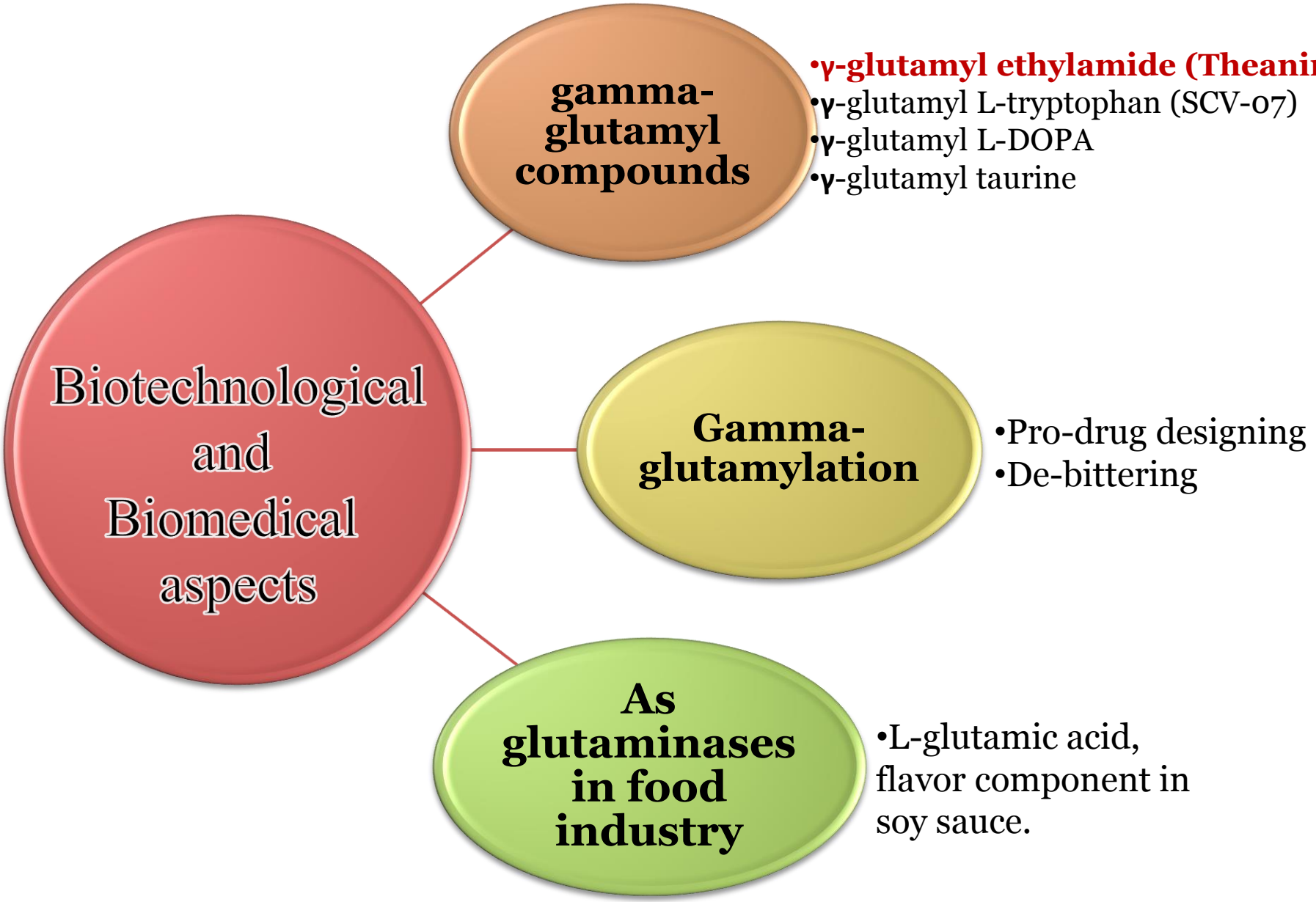


- In plants: no evidence of γ-glutamyl cycle is available while GGT is speculated to be involved in the synthesis of γ-glutamyl compounds.

- Little is known about the physiological role of bacterial GGT, however
  - in *E. coli*, reported to be involved in utilization of glutathione as a nitrogen source.
  - in *Bacillus,* known to play a role in deriving nitrogenous nutrition during limiting conditions.



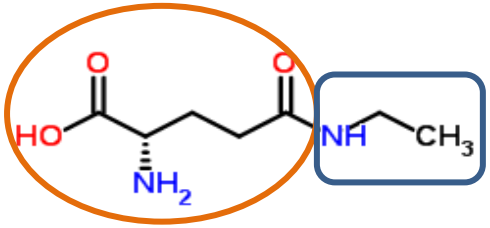
Γ-poly-glutamic acid


Biotechnological and Biomedical aspects
gamma-glutamyl compounds
•γ-glutamyl ethylamide (Theanine)
•γ-glutamyl L-tryptophan (SCV-07)
•γ-glutamyl L-DOPA
•γ-glutamyl taurine
Gamma-glutamylation
•Pro-drug designing
•De-bittering
As glutaminases in food industry
•L-glutamic acid, flavor component in soy sauce.

What is L-theanine??

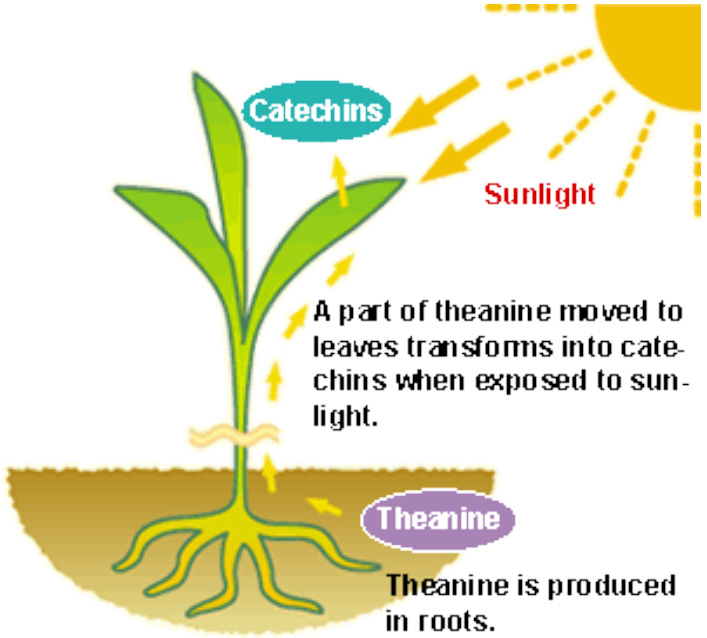


- L-theanine (γ-glutamylethylamide) is a unique amino acid present almost exclusively in the tea plant (*Camellia sinensis).*



L-glutamine Ethylamine

- L-theanine was discovered as a constituent of green tea in 1949 by Sakato, and in 1964 was approved as a food additive in Japan.



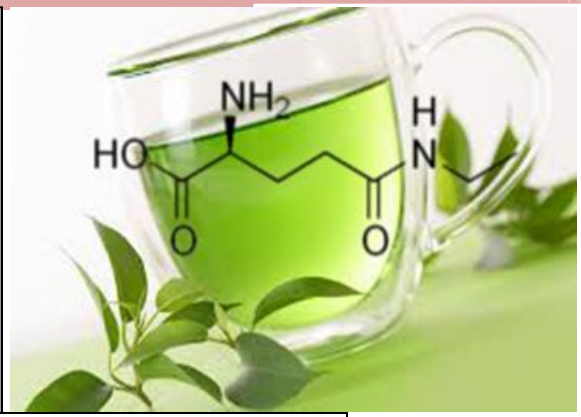
Available online at www.sciencedirect.com
ScienceDirect
Biological Psychology 74 (2007) 39–45
BIOLOGICAL PSYCHOLOGY
www.elsevier.com/locate/biopsycho

L-Theanine reduces psychological and physiological stress responses

Kenta Kimura [a,*], Makoto Ozeki [b], Lekh Raj Juneja [b], Hideki Ohira [a]

[a] Nagoya University Department of Psychology, Chikusa-ku, Nagoya, 464-8601, Japan
[b] Taiyo Kagaku Co., Ltd., 1-3 Takara-machi, Yokkaichi, Mie 510-0844, Japan

Received 6 November 2005; accepted 22 June 2006
Available online 22 August 2006



December 2002 *Biol. Pharm. Bull.* **25**(12) 1513—1518 (2002) 1513

Review

Neuroprotective Effects of the Green Tea Components Theanine and Catechins

Takami KAKUDA

Central Research Institute, Itoen, Ltd.; 21 Mekami, Sagara-cho, Haibara-gun, Shizuoka 421–0516, Japan.

Received April 30, 2002

Available online at www.sciencedirect.com
ScienceDirect
Biological Psychology 77 (2008) 113–122
BIOLOGICAL PSYCHOLOGY
www.elsevier.com/locate/biopsycho

The effects of L-theanine, caffeine and their combination on cognition and mood

Crystal F. Haskell [a], David O. Kennedy [a], Anthea L. Milne [a], Keith A. Wesnes [a,b], Andrew B. Scholey [a,*]

[a] Human Cognitive Neuroscience Unit, Division of Psychology, Northumbria University, Newcastle upon Tyne NE1 8ST, UK
[b] Cognitive Drug Research Ltd., Gatehampton Road, Goring-on-Thames RG8 0EN, UK

Received 28 February 2007; accepted 20 September 2007
Available online 26 September 2007



Brain Waves	Frequency	Mental Condition
Delta wave	0.5 - 3 Hz	deep sleep
Theta wave	4 - 7 Hz	light sleep
Alpha wave	8 - 13 Hz	awake, relaxed
Beta wave	14 Hz	awake, excited




Slow Cow
250 ml
L-Theanine :
Slow Cow ingredient


unwind
Ultimate Relaxation
unwind
Ultimate Relaxation
unwind
Ultimate Relaxation


reduce stress
neuro
BLISS
tropical citrus lychee


RELAX WITH MENTAL CLARITY
VACAY
WATER ENHANCER
RELIEVE STRESS & ANXIETY
EASE TENSION
CALM THE MIND
ALL NATURAL
ISLAND LEMONADE




Day / Night
Relicalm
L-Theanine 150mg
20 Tablets
Assists in reducing nervous tension and anxiousness to provide a calm state and restful sleep

Cipla
WE'LL MAKE IT BETTER


GATORADE
TIGER
FOCUS


DIRECTIONS
INGREDIENTS
CogniTea
Productive Energy
with a hint of mint
FOR A CLEAR AND FOCUSED MIND
100% NATURAL
HERBAL SUPPLEMENT | 1 TEA BAG


JUST
CHILL


01
MUSCLE & STRENGTH
L THEANINE
MYPROTEIN
WWW.MYPROTEIN.COM


Dr's Choice
EasyTime
Relaxing Chocolate Drink
Contains
L-Theanine
No Added Sugar


five stress free
zenify
the five stress free drink
all natural
zen²


ENDOTREX
Citrus Flavor

Conventional sources

- Largely extracted from the leaves of ***Camellia sinensis* (green tea)**.
  - Not eco-friendly and also difficult to meet increasing demand.
- **Chemical synthesis**.
  - Chemically synthesized L-theanine is often not accepted as a food-additive, also the production of racemic mixer of L- and D- forms, high cost and lengthy processing time makes the process unfavorable.

Manufacturers and suppliers

International

- China (Mainland) (2686)
- United States (62)
- Russian Federation (2)
- Armenia (2)
- Canada (3)
- Czech Republic (2)
- Germany (1)
- Hong Kong (2)
- Italy (1)
- Japan (2)
- Macau (1)
- South Korea (4)
- United Kingdom (1)

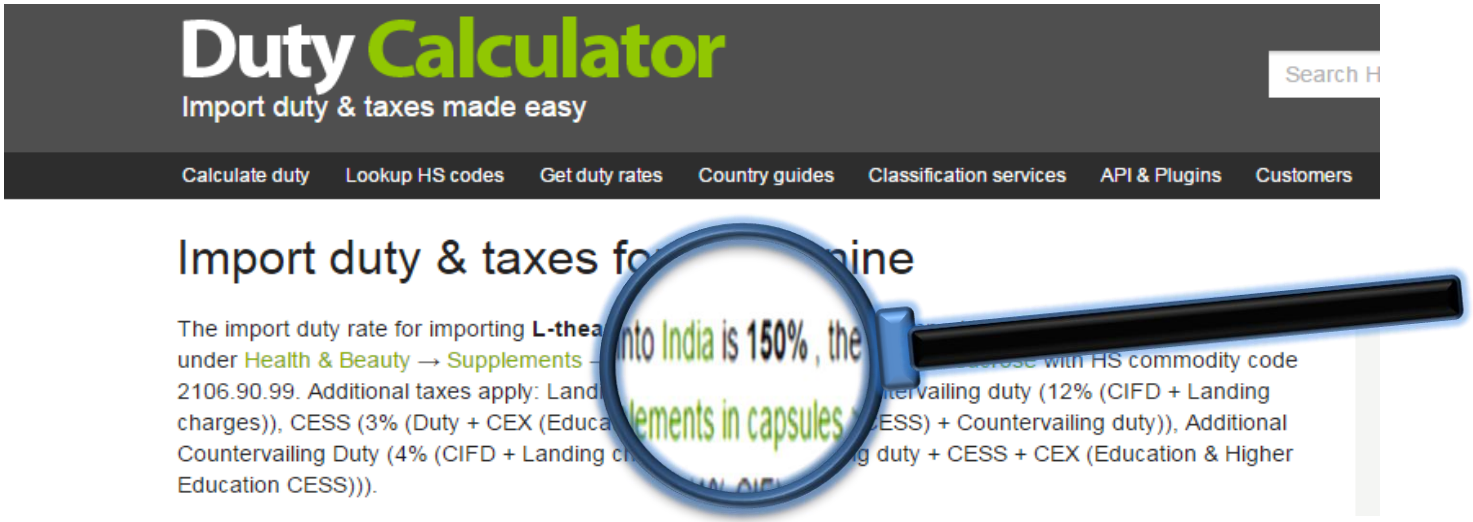
National

- 4 Suppliers
- NO MANUFACTURER



Import and export

- India imported L-theanine worth USD 160,508 with total quantity of 4,832 kg in last year.
- China is the largest supplier of L-theanine accounting for imports worth USD 130,216 followed by Singapore and United States.
- Only Cipla limited has exported a single parcel of its finished product last year to South Africa.

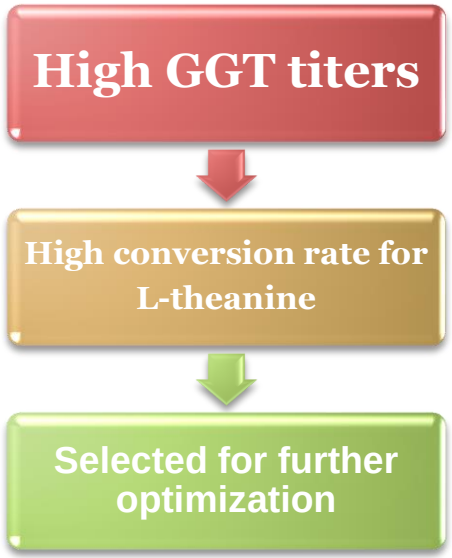


Enzymatic synthesis of L-theanine

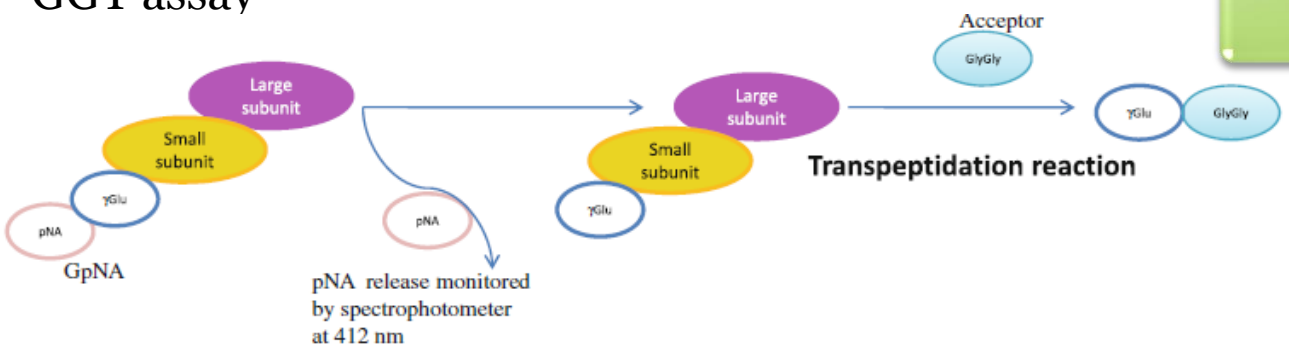
- Enzymatic method of producing L-theanine using bacterial GGT is superior to other methods in various ways:
  - No need of blocking and de-blocking of reactive groups as in chemical reactions.
  - No energy source such as ATP is required because GGT is a transferase and not a synthetase.
  - Bacterial GGT can utilizes less expensive glutamine as well as glutathione.
  - Moreover, bacterial GGTs are either periplasmic or extra-cellular, hence, easier to overproduce and purify.
- Thus, in our laboratory, microbial GGT was employed for the synthesis of L-theanine.

Screening and selection

- Exhaustive screening programme
  - Lab collection of 200 microbial isolates have been screened for the production of GGT enzyme.
  - Also transpeptidation with respect to ethylamine as acceptor has been assessed.



- GGT assay



❖ **The strain selected by above screening procedure was identified to be *Bacillus licheniformis*.**

GGT production optimization

- One-variable at a time
- Statistical methods
  - Plackett-Burman
    - Allow investigation of many factors using few measurments
    - Often used to screen for the important factors that influence process output measures or product quality.
    - Importantly, in PB, the range of each signal variable should be wide.
    - Tells about the effect of each signal factor, but not about the interactions.
  - Response surface methodology
    - the objective is to optimize a response (output variable) which is influenced by several independent variables (input variables) and their interactions.

Composition of minimal medium

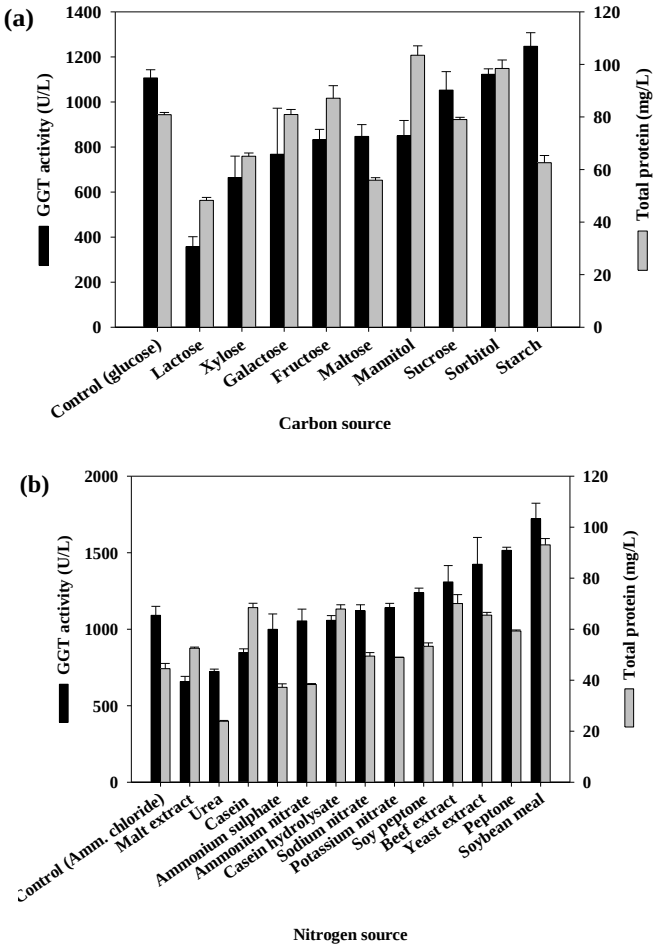
Components	Amount
Na2HPO4	6.4 g/L
KH2PO4	1.5 g/L
NH4Cl	1.0 g/L
NaCl	0.5 g/L
Glucose	4.0 g/L
MgCl2	5.0 mM
Cacl2	0.1 mM

One-variable at a time

GGT activity and total protein obtained after OVAT approach.

Parameter*		**GGT activity (U/L)**	**Total protein (mg/L)**
Temperature (° C)	**37**	**1220.8 ± 82.1**	**105.85± 2.1**
	45	1213.3 ± 59.7	110.96± 0.6
	55	161.7 ± 23.2	51.1± 1.1
Agitation (rpm)	**200**	**1220.8 ± 82.1**	**105.85± 2.1**
	250	1329.2 ± 69.4	85.41± 0.5
	300	1248.9 ± 81.1	86.14± 0.28
pH	**7.0**	**1220.8 ± 82.1**	**105.85± 0.5**
	8.0	1218.8 ± 37.8	105.12± 1.08
	9.0	1120.2 ± 107.3	86.87± 0.47
	10.0	1057.4 ± 48.2	65.7± 1.08
	11.0	97.7 ± 7.5	25.55± 0.3
	12.0	62.8 ± 5.9	36.5± 0.2

Carbon source: Starch
Nitrogen source: Soybean meal
Fold increase: 1.4

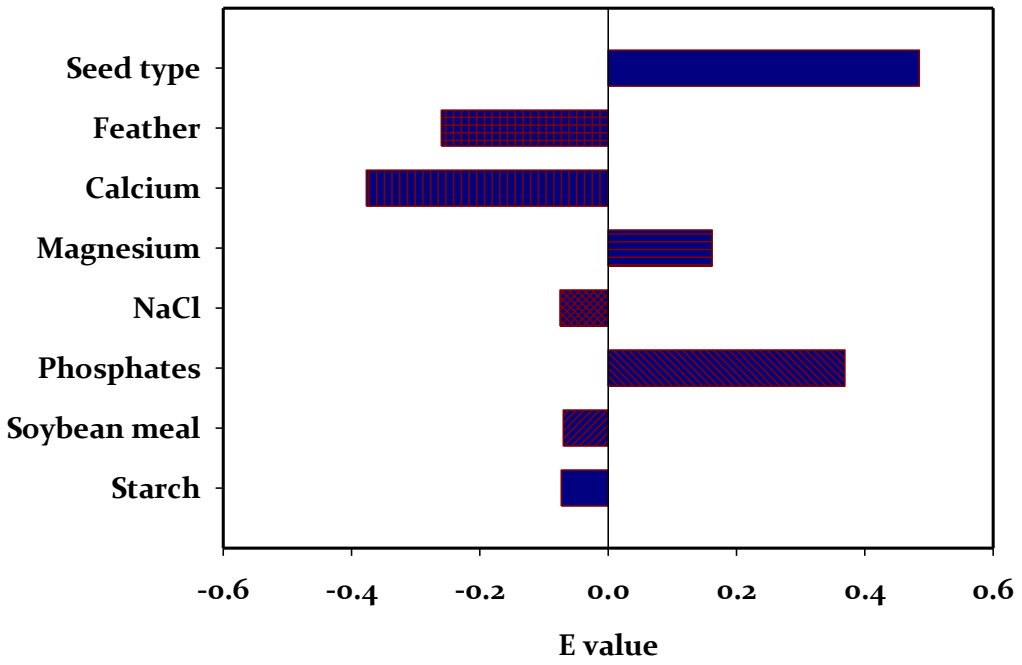


Effect of source of carbon (a) and nitrogen (b) on GGT production from *Bacillus licheniformis* strain.

Plackett-Burman Design

Experimental ranges and levels of all the independent variables used in PB design in terms of actual and coded factors.

Variables	Actual	Coded	Actual	Coded
Starch (% w/v)	0.2	-1	1.0	+1
Soybean meal (% w/v)	0.05	-1	0.5	+1
Phosphates (X strength)	0.5	-1	1.0	+1
Sodium chloride (% w/v)	0.1	-1	5.0	+1
Magnesium chloride (mM)	2.0	-1	10.0	+1
Calcium chloride (mM)	0.2	-1	5.0	+1
Feather (+ or -)	+	-1	-	+1
Seed type*	0.5	-1	1.0	+1

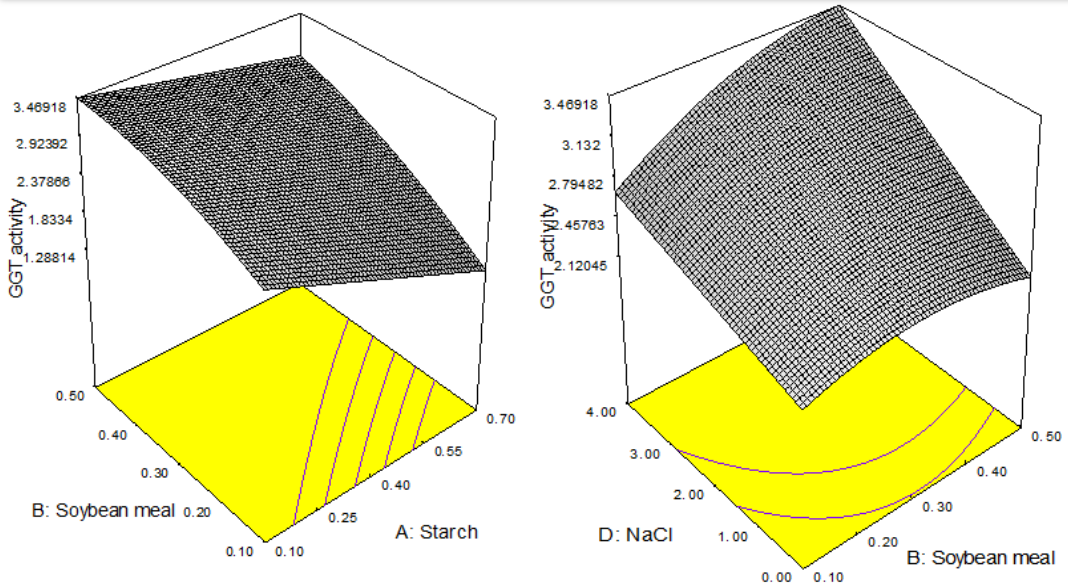


•-1 codes for seed prepared in 12.5 g/L LB medium while +1 codes for Seed prepared in 25 g/L LB medium.

Response surface methodology

Experimental ranges and levels of all the independent variables used in central-composite design in terms of actual and coded factors.

Variables	Actual	Coded	Actual	Coded	Actual	Coded
Starch (% w/v)	0.1	-1	0.4	0	0.7	+1
Soybean meal (% w/v)	0.1	-1	0.3	0	0.5	+1
Phosphates (X strength)	0.5	-1	1.0	0	1.5	+1
Sodium chloride (% w/v)	0	-1	2.0	0	4.0	+1
Magnesium chloride (mM)	5.0	-1	10.0	0	15.0	+1



ANOVA values	Response (GGT activity)
F value	17.13
P > F	< 0.0001
Mean	2.30
R^2	0.8471
Adjusted R^2	0.7977
Predicted R^2	0.6905
Coefficient of variance	6.50
Adequate precision	18.035

Results of centre-composite design (CCD) model in the form of 3D interaction plots along with the predicted values and ANOVA for response surface reduced quadratic model.

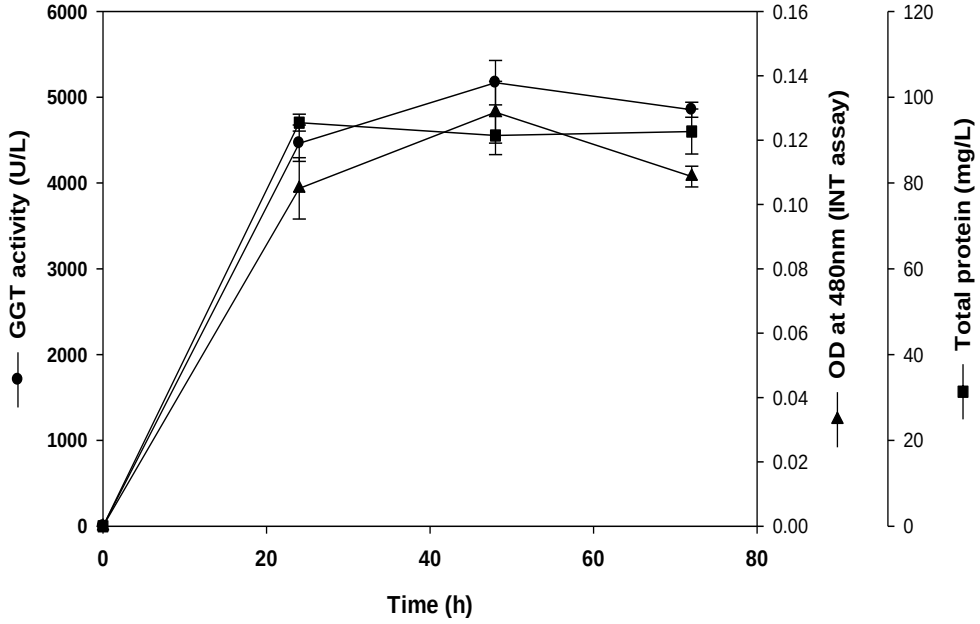
Results of validation experiments

Run No.	Starch (% w/v)	Soybean meal (% w/v)	Phosphates (X strength)	Sodium chloride (% w/v)	Magnesium chloride (mM)	GGT activity (U/L)	
						Actual	Predicted
1.	0.1	0.5	1.0	4.0	5.0	3239.7 ± 194.9	3400
2.	0	0.5	1.0	4.0	5.0	2311.3 ± 221.2	3500
3.	0	0.5	1.0	4.0	0.1	2402.8 ± 107.0	4000
4.	0.1	0.5	1.5	4.0	5.0	3137.5 ± 430.8	3500
5.	0.1	0.8	1.0	7.0	5.0	1282.3 ± 167.9	4500
6.	0.7	0.3	1.0	2.0	15.0	2238.0 ± 206.3	2200
7.	0.7	0.5	1.0	4.0	5.0	2936.9 ± 44.3	2800
8.	0.1	0.5	0.5	4.0	5.0	3347.1 ± 139.6	3300
9.	0.1	0.8	1.0	4.0	5.0	3334.6 ± 163.9	3100
10.	0.4	0.3	1.0	2.0	10.0	2595.4 ± 75.0	2360

- Final medium composition

Components	Amount
Na2HPO4	6.4 g/L
KH2PO4	1.5 g/L
Soybean meal	5.0 g/L
NaCl	40.0 g/L
Starch	1.0 g/L
MgCl2	5.0 mM

Inoculum: 2%
Incubation conditions: 37°C, 200 rpm for 48 h

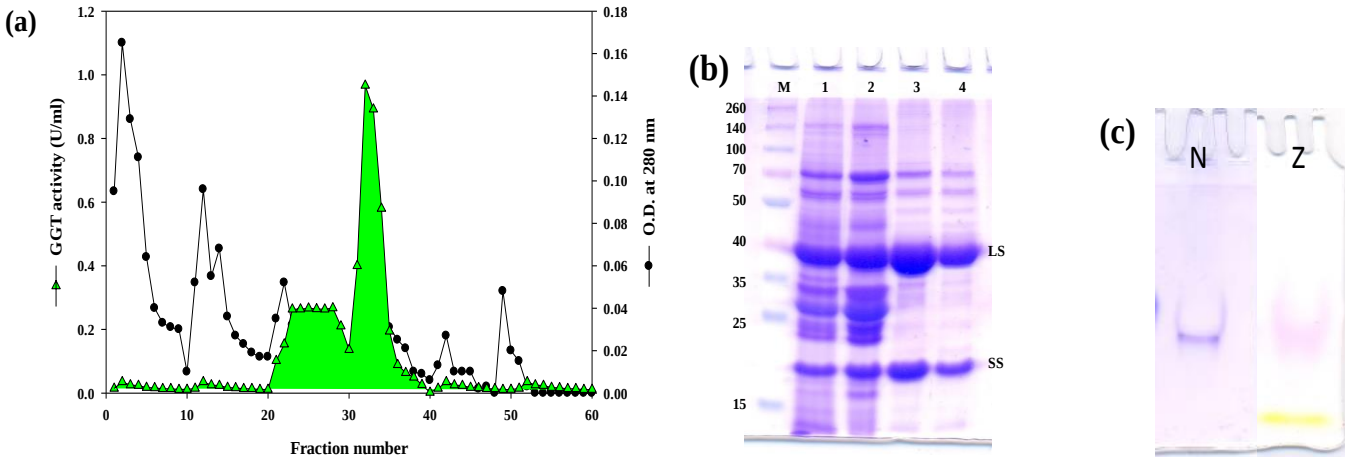


- A total of **5.4 fold** increase in GGT titers from *Bacillus licheniformis* were obtained through medium optimization process.

- At the optimum conditions for enzyme production, a high level, approx. **6500 U/L** of GGT was obtained.

- A maximum of 3200 U/L has been reported from *Bacillus subtilis* NX2.

GGT purification



Purification profile (a), SDS-PAGE* (b) and native-PAGE along with zymogram (c) of wild GGT from *Bacillus licheniformis* strain.

* M: Marker; 1: crude; 2: 100KDa retentate; 3: 10KDa retentate; 4: IEC purified fraction.

Purification scheme for wild GGT from *Bacillus licheniformis* strain.

Step	Total GGT activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification yield (%)	Purification fold
Crude	4,109	13.3	308.94	1	1
Ultra-filtration (100KDa)	3,320	11.6	286.20	80.79	0.92
Ultra-filtration (10KDa)	2,077	3.25	639.07	50.5	2.06
Q-sepharose IEC (0.4 M NaCl fraction)	1,530.45	2.36	648.49	37.2	2.09

GGT immobilization

Entrapment method

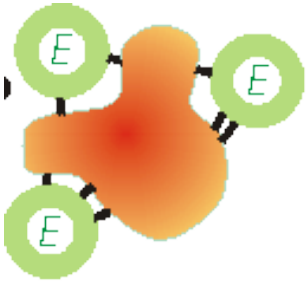
- **Ca-alginate immobilization**



- Weak binding
- Enzyme leakage is high
- Lesser recycling efficiency

Covalent immobilization

- **Immobilization on chitosan microsphere**



- Strong binding
- Enzyme leakage is negligible
- High recycling capability

Immobilization pH, enzyme concentration and time were standardized in order to have maximum enzyme to be immobilized on CMS.

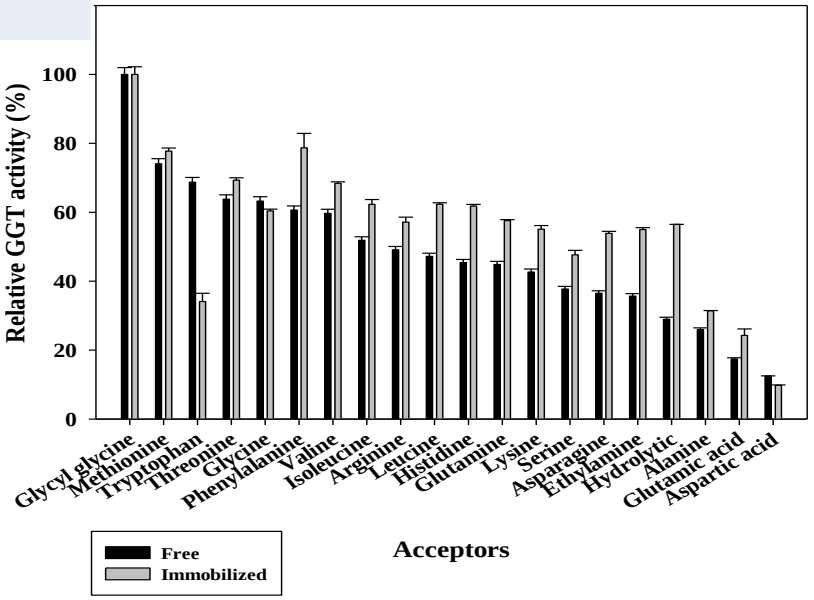
Parameters	Immobilization (%)
pH (Temp: 18°C; CT: 16h; E: 65mg)	
7.0	97.9 ± 1.8
8.0	97.6 ± 2.2
9.0	**97.9 ± 2.4**
10.0	68.6 ± 1.7
11.0	58.2 ± 2.0
Enzyme* (pH: 9.0; Temp: 18°C; CT: 16h)	100 ± 2.9
0.2 (1 ml)	**94.4 ± 2.1**
0.4 (2 ml)	49.2 ± 1.8
0.8 (4 ml)	
Coupling Time (pH: 9.0; Temp: 18°C; E: 1.2 mg)	89.6 ± 1.3
2 h	99.5 ± 1.7
3 h	**99.7 ± 1.1**
4 h	

* Specific activity of GGT is 101.0 U/mg with the protein content of 0.2 mg/ml. Coupling time was estimated by taking 0.4 mg protein.

Comparative biochemical characterization

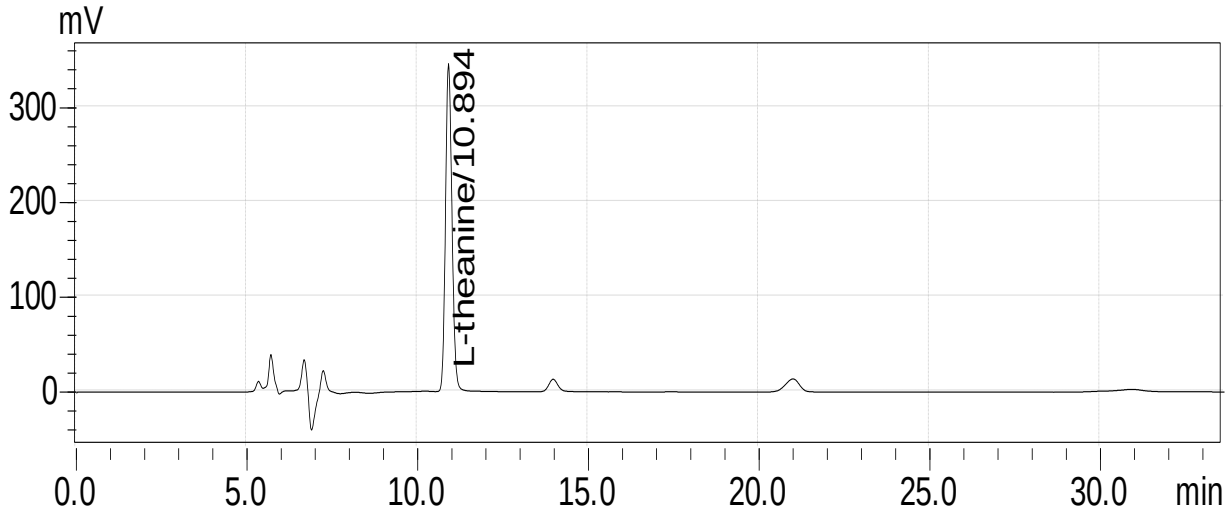
Parameter	Free	Immobilized
Temperature optima	60°C	60°C
pH optima	9.0	9.0
Thermal stability	@50°C $t_{1/2}$= 170min @60°C $t_{1/2}$= 6min	**@50°C $t_{1/2}$= 450min** **@60°C $t_{1/2}$= 50min**
pH stability	6.0-11.0	6.0-11.0
Inhibitors	EDTA, NBS, DON and azaserine	EDTA, NBS, DON and azaserine
Activators	nil	Ca^{2+} and Cu^{2+}

Immobilized enzyme showed better affinity towards various acceptors



L-theanine: HPLC detection

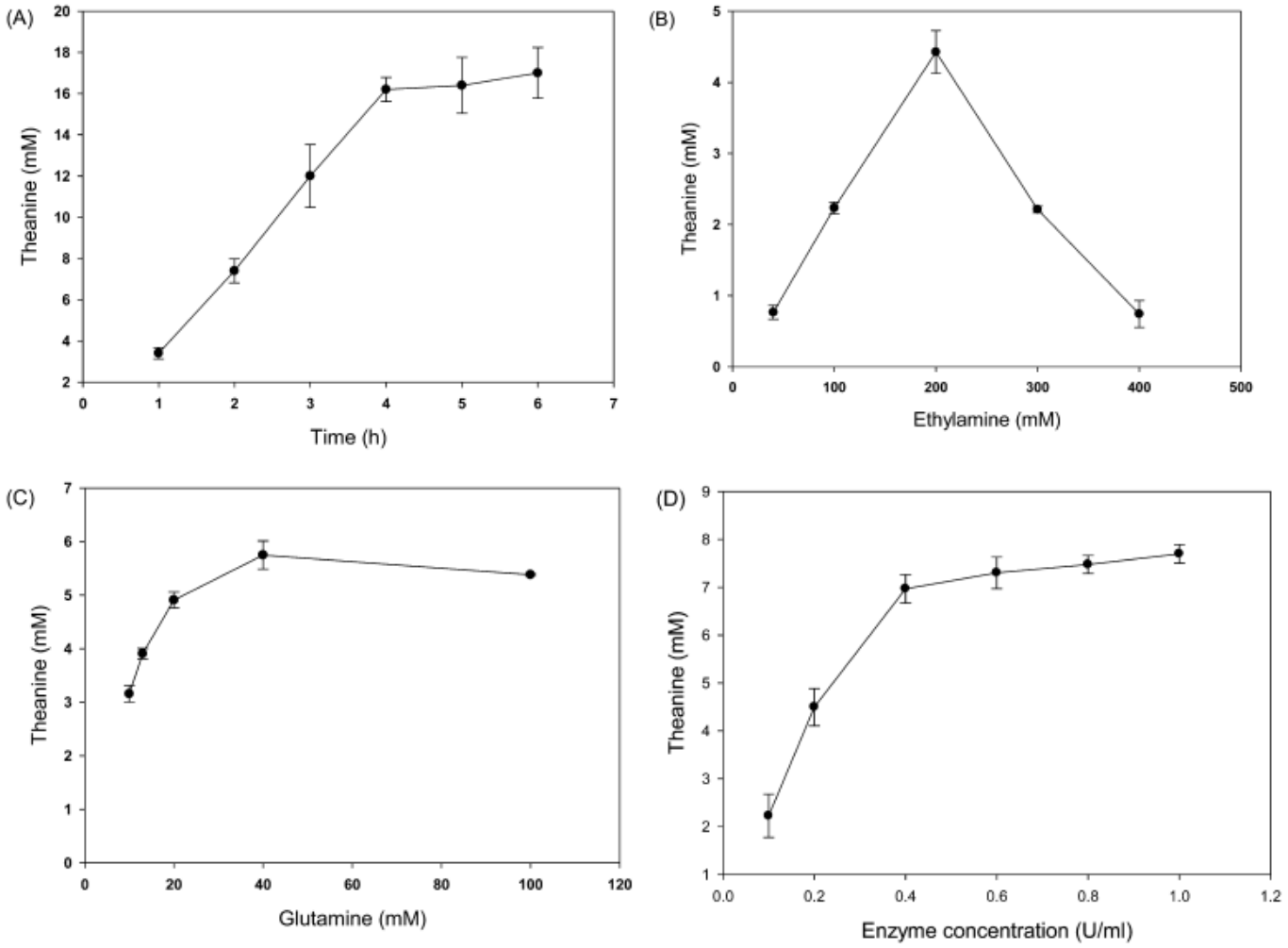
- Simple detection method was employed requiring no pre or post-column derivatization.
- Mobile phase: 0.05% trifouroacetic acid (TFA)
- Flow rate: 0.5 ml/min
- Detector: UV/vis
- Detection at 203 nm
- Retention time: 10.8 – 11.0



Parameters optimization

- Parameters effecting L-theanine synthesis:
  1. pH
  2. Temperature
  3. Donor to acceptor ratio
  4. Enzyme concentration
  5. Time of reaction

- Initially all the parameters were optimized using free enzyme.



(A) Time profile for L-theanine synthesis (reaction containing 20mML-glutamine, 200mMethylamine, and 0.4 U/mL BLGGT in Tris-Cl, pH 9.0, buffer (50 mM), kept at 37 °C). (B) Optimization of acceptor concentration by a one variable at time approach (L-glutamine was kept constant at 20 mM. (C) Effect of L-glutamine concentration (ethylamine concentration was kept constant at 200 mM). (D) Optimization curve of enzyme concentration for theanine synthesis (5 mL reaction containing 40 mM L-glutamine and 200 mM ethylamine). All of the above reactions were done in triplicate and were carried out at 37 °C and pH 9.0 for 1 h.

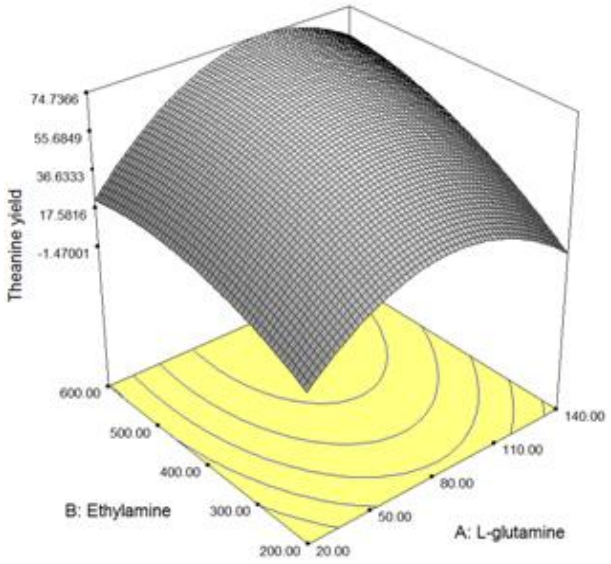
Box-Behnken design and the results along with the variance analysis for the selected quadratic model.

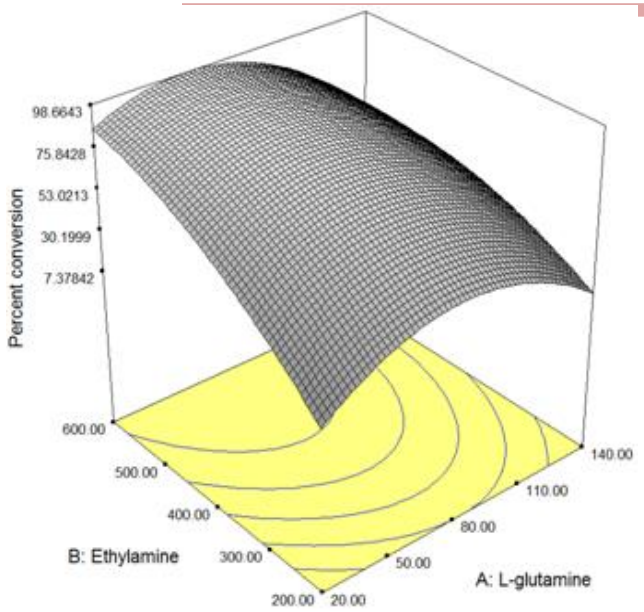
(a)

Run No.	L-glutamine (mM)	Ethylamine (mM)	BLGGT (U/mL)	Theanine yield (mM)		Percent conversion (%)	
				Actual	Predicted	Actual	Predicted
1	0	-1	+1	17.09	21.99	21.36	28.84
2	-1	0	-1	2.45	4.04	12.24	31.45
3	0	-1	-1	12.31	15.75	15.38	10.19
4	+1	-1	0	10.41	5.49	7.43	7.54
5	0	+1	+1	68.88	60.88	86.1	85.59
6	+1	0	-1	31.02	26.32	22.16	9.92
7	0	0	0	65.63	63.42	82.04	79.27
8	-1	+1	0	17.18	22.1	85.92	85.82
9	+1	+1	0	56.27	59.7	40.19	42.59
10	0	0	0	62.51	63.42	78.13	79.27
11	+1	0	+1	26.37	32.56	18.83	28.57
12	0	0	0	58.42	63.42	73.03	79.27
13	-1	-1	0	1.95	-1.47	9.77	7.38
14	0	+1	-1	54.97	54.64	68.71	66.93
15	-1	0	+1	13.36	10.28	66.82	50.11
16	0	0	0	67.12	63.42	83.9	79.27
17	0	0	0	63.41	63.42	79.26	79.27

(b)

ANOVA values	Response	
	Theanine yield	Percent conversion
F value	45.38	18.48
P > F	< 0.0001	0.0001
Mean	37.02	50.08
R2	0.9724	0.9350





On the basis of the regression analyses following equations were generated for Y_1 and Y_2:

Theanine yield (Y_1) = $+63.42 + 11.14.\ X_1 + 19.44.\ X_2 + 3.12.\ X_3 - 30.99.\ X_1^2 - 10.97.\ X_2^2 - 14.13.\ X_3^2 + 7.66.\ X_1.\ X_2$

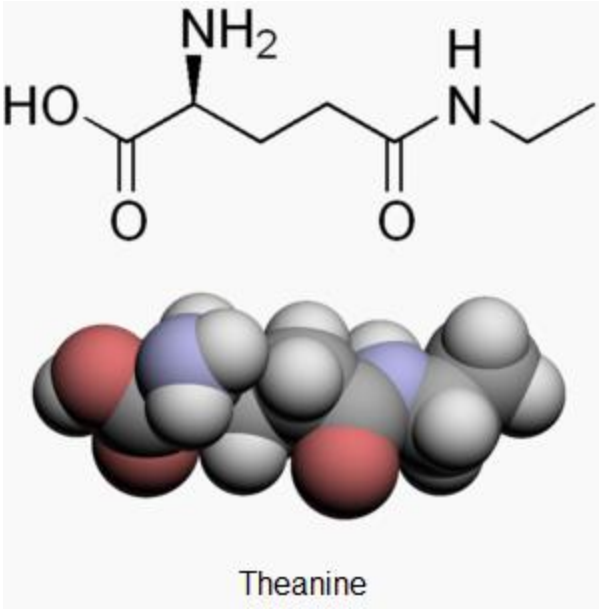
Percent conversion (Y_2) = $+79.27 - 10.77.\ X_1 + 28.37.\ X_2 + 9.33.\ X_3 - 30.66.\ X_1^2 - 12.78.\ X_2^2 - 18.60.\ X_3^2 - 10.85.\ X_1.\ X_2$

where X_1, X_2 and X_3 are three independent variables included in the study. The interacting parameters, X_{13} and X_{23} were found to be insignificant and thus excluded from the model.

Finally optimized reaction condition

- 80 mM L-glutamine,
- 600 mM ethylamine, and
- 1 U/mL BLGGT

at pH 9.0 and 37 °C for 2 h



Theanine

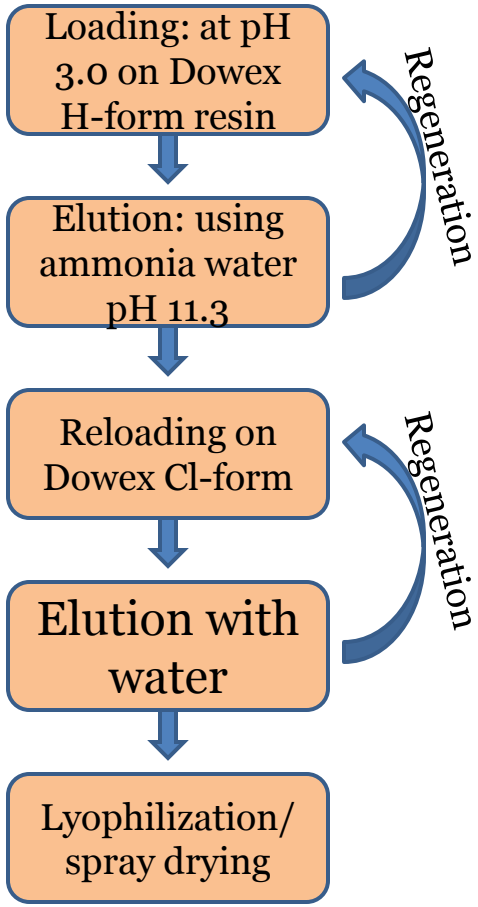
- Conversion rate: **85-87%**
- Theanine yield: 68-70 mM
- **4.25 fold** of the initial yield

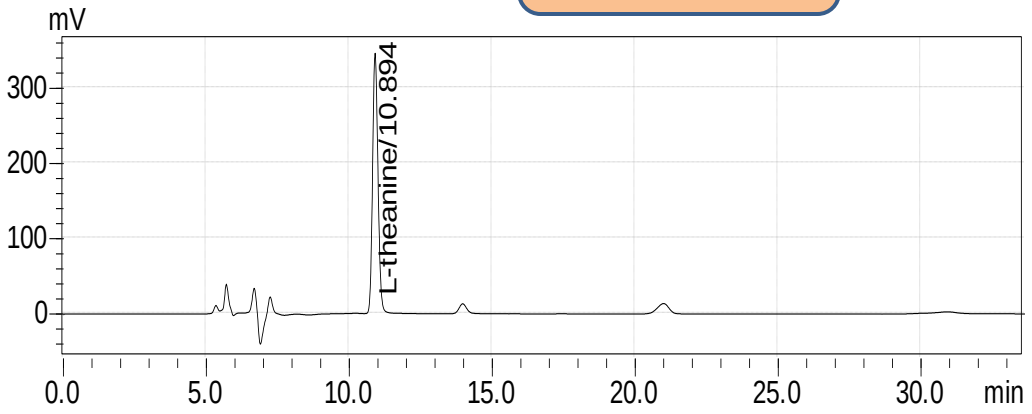
- Under optimized conditions 84% conversion was achieved using immobilized enzyme in 50 mL of reaction volume within 2 h, which was scaled up to 1 L with similar yields.

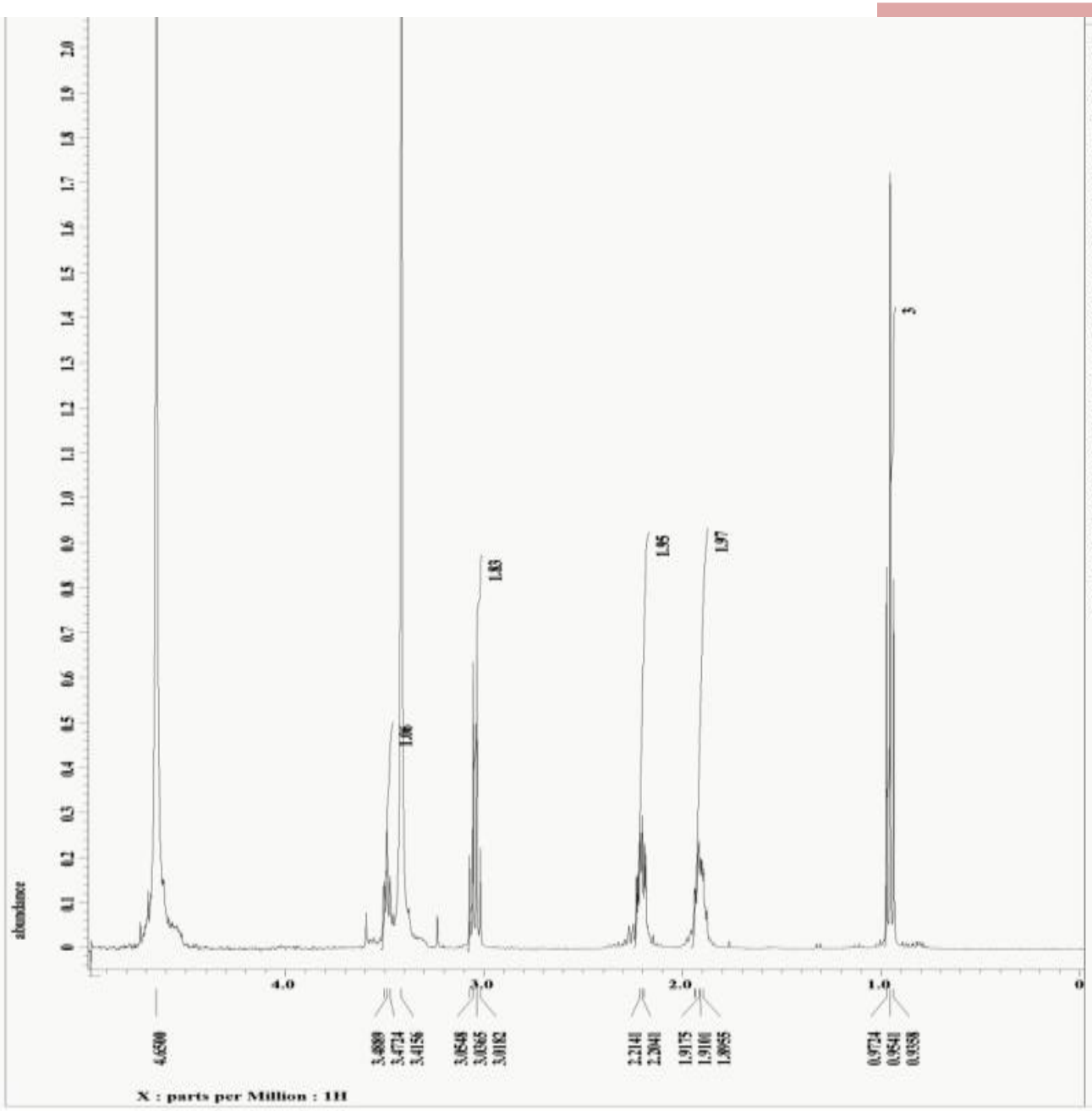
- Chitosan microsphere immobilized GGT was reused for 10 times with >90% efficiency retained in every cycle.

Purification of L-theanine

- L-theanine was purified by the method of Zhu *et al.* (2007) with few modifications.
- More than 90% of pure L-theanine was recovered by this purification protocol which is around 12 g per cycle.
- The purified fractions were then subjected to lyophilization and finally a white colored theanine powder was obtained.
- Purity of the final product was assessed by HPLC and H^1-NMR








```
Filename          = RNG_TE_PROTON-
Author            = delta
Experiment        = single_pulse.
Sample_id         = RNG_THE
Solvent           = D2O
Creation_time     = 27-AUG-2013 1
Revision_time     = 21-JUL-2014 1
Current_time      = 21-JUL-2014 1

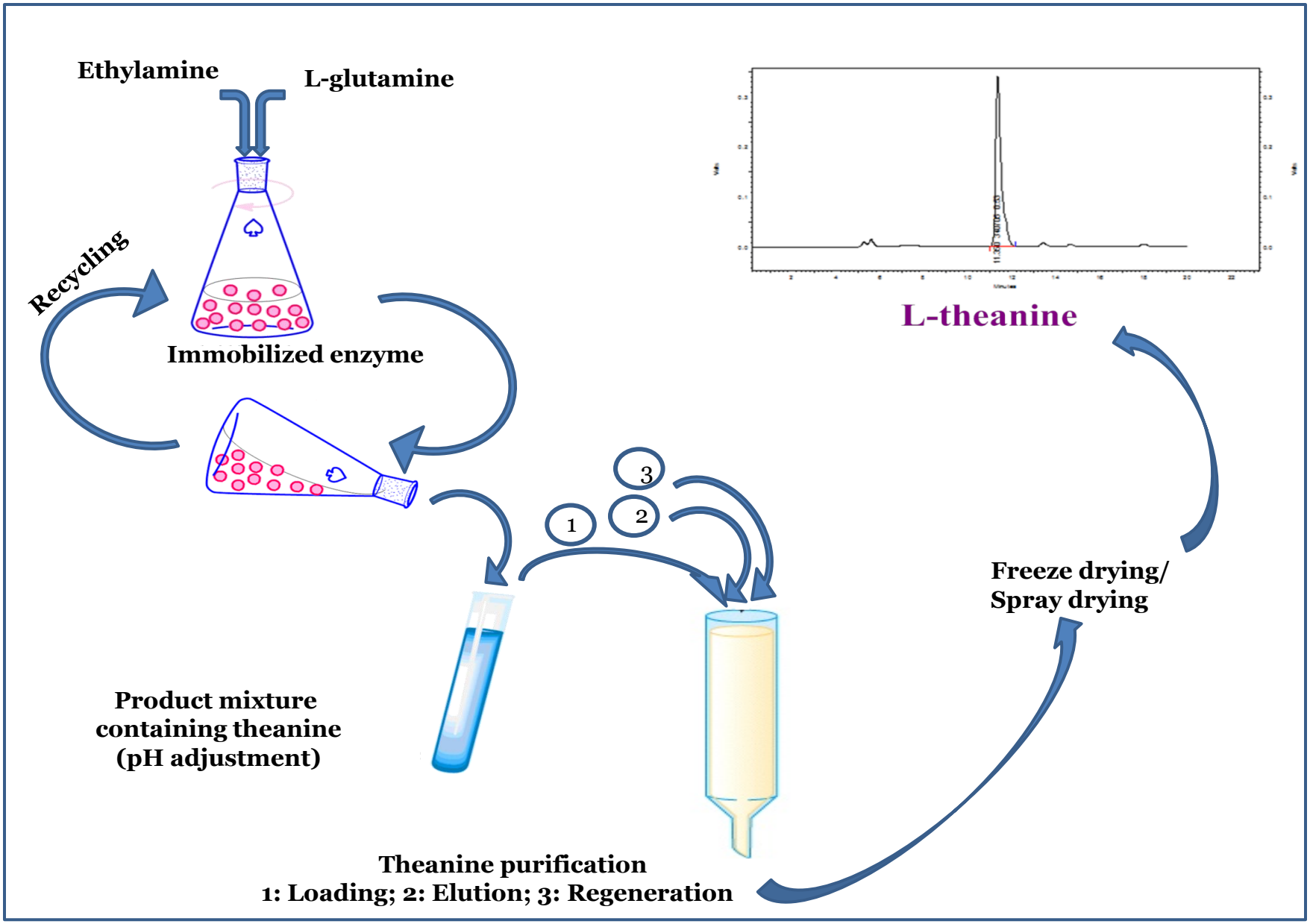
Comment           = RNG THE
Data_format       = 1D COMPLEX
Dim_size          = 13107
Dim_title         = 1H
Dim_units         = [ppm]
Dimensions        = X
Site              = ECX 400P
Spectrometer      = DELTA2_NMR

Field_strength    = 9.389766[T]
X_acq_duration    = 1.36577024[s]
X_domain          = 1H
X_freq            = 399.78219838[
X_offset          = 5[ppm]
X_points          = 16384
X_prescans        = 1
X_resolution      = 0.73210757[Hz]
X_sweep           = 11.99616123[k
Irr_domain        = 1H
Irr_freq          = 399.78219838[
Irr_offset        = 5[ppm]
Tri_domain        = 1H
Tri_freq          = 399.78219838[
Tri_offset        = 5[ppm]
Clipped           = FALSE
Mod_return        = 1
Scans             = 16
Total_scans       = 16

X_90_width        = 11.31[us]
X_acq_time        = 1.36577024[s]
X_angle           = 45[deg]
X_atn             = 4.5[dB]
X_pulse           = 5.655[us]
Irr_mode          = Off
Tri_mode          = Off
Dante_presat      = FALSE
Initial_wait      = 1[s]
Recvr_gain        = 34
Relaxation_delay  = 4[s]
Repetition_time   = 5.36577024[s]
Temp_get          = 23.7[dC]
```

Comparative analysis of the methods available for enzymatic synthesis of L-theanine using GGT from various sources.

Γ- glutamyl donor used	Enzyme	Donor concentration (mM)	Acceptor (Ethylamine) concentration (mM)	Conditions	Time (h)	Conversion (%)	Reference
L-glutamine	**B.licheniformis GGT (BLGGT)**	**80**	**600**	**pH 9.0, 37 °C**	**2**	**≥ 84 in each cycle**	**Current study**
	Recombinant E.coli GGT	200	1500	pH 10.0, 37 °C	5	60	Suzuki et al., 2002
	Bacillus subtilis GGT	20	50	pH 10.0, 37 °C	4	94	Shuai et al., 2010
	Recombinant E.coli GGT	267	2000	pH 10.5, 37 °C	24	80	Wang et al., 2011
Glutamic acid γ-methyl ester (GAME)	Immobilized E.coli cells	300	3000	pH 10.0, 50 °C	18	87.2 after 6 cycles	Zhang et al., 2010
	E.coli cells	100	1000	pH 10.0, 45 °C	8	95	Zhang et al., 2010
γGpNA	Recombinant E.coli GGT	5	50	pH 9.0, 37 °C	6	93	Zhang et al., 2013


Ethylamine
L-glutamine
Recycling
Immobilized enzyme
L-theanine
3
1
2
Freeze drying/
Spray drying
Product mixture
containing theanine
(pH adjustment)
Theanine purification
1: Loading; 2: Elution; 3: Regeneration

Conclusive remarks

- 1 L fermentation results into 6500 U of GGT enzyme.
- 6500 U of enzyme requires approx. 200 g (wet weight)of CHITOSAN MICROSPHERES (CMS).
- CMS immobilized enzyme can be recycled for more then **10 cycles**.
- 12 g/L of L-theanine requires 5% (wet weight/v) of CMS-GGT. Total yield of 120 g in 10 cycles.

Therefore, 1 L enzyme production would yield around **480 g** of L-theanine.

WHAT NEXT

- Up-scaling of the process.
- Strain improvement.
- Protein engineering
  - Heterologous expression has been standardized
  - *In-silico* analysis of protein is being undertaken.

Thank you
& enjoy tea……☺

